

The effect of antimicrobial photodynamic therapy against virulence genes expression in colistin-resistance *Acinetobacter baumannii*

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Background and Aims: The emergence of drug-resistant infections is a global problem. *Acinetobacter baumannii* has attracted much attention over the last few years because of resistance to a wide range of antibiotics. Applying new non-antibiotic methods can save lives of many people around the world. Antimicrobial photodynamic therapy (aPDT) technique can be used as a new method for controlling the infections. In this study we investigated the effect of aPDT on the expression of pathogenic genes in colistin-resistance *A. baumannii* isolated from a burn patient.

Materials and methods: The suspension of colistin-resistance *A. baumannii* was incubated with 0.01 mg/ml of toluidine blue O (TBO) in the dark; then the light emitting diode device with a wavelength of 630 ± 10 nm and output intensity of 2000-4000 mW /cm² was irradiated to the suspension at room temperature. Subsequently, after the aPDT, genes expression of *ompA* and *pilZ* was investigated by using real-time polymerase chain reaction technique.

Results: Among the genes studied, the transcript of the *ompA* gene after aPDT was increased significantly in comparison with control groups ($P < 0.05$). Whereas, there was no remarkable different in *pilZ* gene expression ($P > 0.05$).

Conclusions: It can be concluded from the results that the *ompA* as an outer membrane of *A. baumannii* is degraded after exposing aPDT and it will probably be done the penetration of antibiotics into cells of this bacterium easily.

Key words: *Acinetobacter baumannii* • antimicrobial photodynamic therapy • toluidine blue O • LED • colistin

Introduction

Recently, *Acinetobacter baumannii* has been proposed as one of the most important opportunistic pathogens in burn patients. The appearance of multiple drug -resistant

(MDR) strains of *A. baumannii*, force physicians to use polymyxin (e.g. colistin) in the treatment of infections caused by this microorganism. Therefore, that results in increasing treatment costs and additional therapies ¹⁾.

In the 1970s, this bacterium was highly sensitive to various antibiotics, but currently is the main cause of drug-resistant nosocomial infections. This bacterium accounts for 2 up to 10% of total hospital infections caused by Gram-negative bacteria ²⁾. Appearance of MDR, XDR (extensively drug-resistant) and PDR (Pandrug-resistant) strains and increased hospital infections due to this strain

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of bacteria have attracted more attention in recent years. PDR strains are also resistant to polymyxin and tigecycline³⁾.

A. baumannii does not produce special toxins or cytotoxins. Few virulence factors have been identified up to now. In fact, *A. baumannii* is able to accumulate diverse resistance mechanisms, innate resistance to antiseptics leading to survive in stressful environmental conditions (such as food shortages and high density of bacteria). These characteristics have introduced *A. baumannii* as a highly resistant opportunistic pathogen in the hospital⁴⁾.

OmpA (Outer membrane protein A), one of the outer membrane proteins of *A. baumannii*, works as a critical virulence factor. This factor is involved in the development of biofilm as well as bacteria and human cell interactions, facilitating invasion of human epithelial cells and induce apoptosis in human laryngeal epithelial cells. This bacterium can survive in the bloodstream by the OmpA^{5,6)}.

Although, *A. baumannii* is nonmotile, but it can quickly spread on surfaces with twitching motility phenomena. *pilZ* gene is one of the main genes playing significant role in control and synthesis type IV pili. Also, this gene has been identified as one of the main biofilm producers^{7,8)}.

It is crucial to find proper treatment for *A. baumannii* for several reasons such as significant prevalence of *A. baumannii*, easy contamination, widespread infections and resistance to most of current antibiotics (even polymyxin)³⁾.

Nowadays, a lot of international research efforts are underway for discovering new antimicrobial agents or an alternative antimicrobial method. Antimicrobial photodynamic therapy (aPDT) is one of the recent antimicrobial methods to deal with such infections⁹⁾. aPDT selectively targets cells growing quickly or hyperproliferating, hence, because of their rapid growth microorganisms can be one of the objectives for aPDT¹⁰⁻¹²⁾. aPDT might indicate an excellent alternative or additional therapy for the treatment of local infections which represent resistance to antibiotic drugs, because it is recognized as a safe treatment strategy that is both minimally invasive and nontoxic¹³⁾.

aPDT is a technique containing a photosensitizer combined with light of a specific wavelength which produces cytotoxic elements such as singlet oxygen and free radicals. Consequently, these products cause some damages in essential cell components or modify metabolic activities irreversibly leading to bacterial cell death¹⁴⁾.

Although variety of studies show aPDT inhibitory effect on bacterial growth, little information is available about aPDT cellular and molecular effects on bacteria remaining after exposure to low radiation doses. Genomic studies might lead to better understanding of molecular changes on bacteria exposed to aPDT.

In this study we investigated molecular effects of aPDT on expression of genes *ompA* and *pilZ* in *A. baumannii* to get more information about molecular function of aPDT in physiological and metabolically process in bacteria.

Material Method

Bacterial strains and culture conditions

Colistin-resistant *A. baumannii* strain isolated from burn-wound infection was used. The phenotype of *A. baumannii* is defined as XDR consistent with the International Expert Proposal for Interim Standards Guidelines¹⁵⁾. This strain is resistant to colistin, and the minimal inhibitory concentration (MIC) value of colistin was > 32 µg/ml.

First *A. baumannii* was cultured on the Brain Heart Infusion (BHI) broth (CONDA, Spain) culture media under aerobic condition at 37°C for 24 h. One ml bacterial suspension was added into 10 ml fresh BHI broth then specimens were incubated with shaking incubator at 100 rpm under aerobic condition at 37°C until the bacteria have reached a log phase; were adjusted to a concentration of 1.0×10^6 colony forming units (CFU)/ml, as verified by both spectrophotometry (optical density [OD] 600: 0.01 – 0.02) and colony counting.

Photosensitizer and light source

Toluidine blue O (TBO) was used as a photosensitizer (PS). TBO powder (Merck, Germany) was solved in sterile normal saline to prepare the concentration of 0.01 mg/ml of TBO. The TBO solution was sterilized by a 0.22-micron syringe filter and stored at 4°C in the dark before use; Also in this study light emitting diode (LED) with a wavelength of 630 ± 10 nm and output intensity of 2000-4000 mW/cm² was used¹⁶⁾ as a light source to activate the PS. The distance between the light source and each well surface was fixed at 1 mm.

Study design for evaluation of target genes expression

Expression of *ompA* and *pilZ* genes in studied strain was evaluated after aPDT. In the PS group (photosensitizer), microbial suspension was mixed only with the PS in the darkness, and in L group (LED irradiation), microbial suspension was only irradiated with LED light irradiation. In C group (control) microbial suspension was not exposed to any irradiation and PS material. After 10 min of incubation, gene expression was evaluated in PS, L and C groups. Microbial suspension in group L+ PS was exposed to PS and LED and then gene expression was assessed. All experimental were repeated for three times. Groups which were evaluated for expression of target genes are classified in **Table 1**.

Exposure of the colistin-resistant *A. baumannii* strain with aPDT

To prepare microbial suspension for groups C and L, 2 ml of bacterial suspension was centrifuged for 40 s at 14,000 × g, cell pellet in 400 µl of sterilized normal saline was suspended. In the case of PS and L + PS groups, as in previous groups, 2 ml of the microbial suspension was centrifuged for 40 s at 14,000 × g cell pellet was suspended in 200 µl of sterilized normal saline. Subsequently, for binding of PS to bacteria (cells), samples with 200 µl TBO at 0.01 mg/ml concentration were shaken on the Thermo Mixer Shaker (manufactured by Eppendorf Company, Germany) for 10 min (pre-irradiation time) in darkness and at room temperature.

After incubation in the dark, the wells were irradiated by a LED device with the wavelength of 630 ± 10 nm for 60 s. Radiation was performed under the sterile conditions in the laminar flow in a dark room. The distance between the tip of the LED to the sample was 4.5 mm and the depth of the wells was 10 mm. To prevent the spreading of light to adjacent samples, around wells were filled with TBO dye. The wells contained colistin-resistant *A. baumannii* suspension that was not exposed to any radiation and no light sensitive material (PS) was as a control.

RNA extraction from colistin-resistant *A. baumannii* treated with aPDT

Immediately after aPDT, the microbial suspension was transferred to the RNase free microtube and the extraction process was done according to the manufactur-

er's instructions easy-spin (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology Company, Korea). The total RNA samples obtained were treated with DNase I (Thermo Scientific GmbH, Germany) after assessment with NanoDrop spectrophotometer (Thermo Fisher Scientific, US). The concentration of total RNA was determined using a ultraviolet absorbance at 260 nm (A260) and 280 nm (A280) with NanoDrop spectrophotometer. The concentration of total RNA must be within an A260 / A280 ratio of 1.80 – 2.00 to ensure the integrity of the mRNA. The integrity of the RNA was determined by agarose gel 1% electrophoresis. The extracted RNA was used as a template to synthesize the primary cDNA chain by reverse transcription with a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, US) following the manufacturer's instructions. The cDNA was stored at -20°C.

Real-time PCR

The resulting cDNAs were used for the quantification of virulence genes mRNA levels by real-time qRT-PCR with Line-Gene K Real-Time PCR Detection System (Hangzhou, China).

All primers utilized in this study were designed by using the Primer3Plus web tool (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>). The PCR primer sequences used for quantification of the candidate reference is given in **Table 2**. The same primer pair was used when determining the mRNA level and DNA copy number of a certain gene. Gene-specific amplification was confirmed by a single peak in melt-curve analysis. The PCR products were subjected to electrophoresis in 2% (w/v) agarose gels to confirm the specificity of ampli-

Table 1: Groups evaluated for expression of *ompA* and *pilZ* genes.

Study design	
Group C	Microbial suspension was not exposed to any irradiation and PS material.
Group L	Microbial suspension was only irradiated with LED light irradiation.
Group PS	Microbial suspension was mixed with the PS in the darkness.
Group L+ PS	Microbial suspension in group L+ PS was exposed to TBO and LED irradiation.

Table 2: The sequences of primers used in this study.

Gene	Forward primer sequence (5' - 3')	Reverse primer sequence (3' - 5')
<i>ompA</i>	AGCATAAAGAAGCTACACCTGC	AAAGTCGCCAAGAAACCTTGAT
<i>pilZ</i>	AGCAAAAAGTGAAGATGGGTCA	ACCTTGCGGTTTAAATCCACTT
<i>16SrRNA</i>	AAAGTTGGTATTTCGCAACGG	ACCTTTAACCCGCTTTTGCT

fication and the absence of primer dimer formation. Amplification was followed by melting-curve analysis to check the specificity of the PCR product.

The reaction volume of 20 μ l contained 10 μ l of SYBR Premix Ex Taq II (Takara, Japan) under the following conditions: 95°C for 5 min, amplification for 35 cycles with denaturation at 95°C for 15 s, annealing for 10 s at the temperature reported and extension at 72°C for 10s. The specificity of the primers was evaluated using melt curves.

The relative amounts of each target gene (*ompA*, *pilZ*) were quantified (qPCR) relative to *16S rRNA* (as an internal standard). The fold changes of the target genes' expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method using the Relative Expression Software Tool (REST) 2009 software (QIAGEN)¹⁷. The mRNA expression levels were shown as n-fold differences relative to the calibrator. Changes in expression levels of the target genes were interpreted as significant if the variation was > 2-fold.

Statistical analysis

ANOVA and Tukey's test was employed for statistical analysis. T-test were used to assess the difference between the relative quantities of the gene expressions in *A. baumannii* strains under the treatments. All experiments were performed in triplicate, and results were reported as mean $\pm$ standard deviation (SD). P-values less than 0.05 in all experiments were considered statistically significant.

Result

As you see in **Figure 1**, of the two genes studied in the L+ PS group, the transcription of the *ompA* gene increased considerably compared to the C, L and PS groups ($P < 0.05$). On contrast, there was not any significant change shown in the expression of the data obtained from the results of the analysis of the *pilZ* gene in the L+ PS group. As in all studied groups, the level of expression of this gene was similar to control group ($P > 0.05$; **Fig. 1**). According to the results, the level of *ompA* gene expression was significantly different versus *pilZ* in L+ PS group ($P < 0.05$), so that it was able to increase the expression of *ompA* gene by 8.7-fold more than *pilZ*.

Discussion

A. baumannii is known as one of the most important common agent of health care-associated infections which leads to intractable infections such as burn infections, respiratory infections (pneumonia), infections caused by the ventilator (Ventilator-Associated Pneumonia), wound and soft tissue infections, urinary tract infection, meningitis and bacteremia particularly in patients admitted to the intensive care unit (ICU) and the burn ward of a hospital¹⁸. Its ability of resistance to antibiotics is very strong resulting in emergence of MDR strains¹⁹. Worldwide distribution of strains resisted to various classes of antibiotics is worrying and leads to higher medical expenses⁷. Recent-

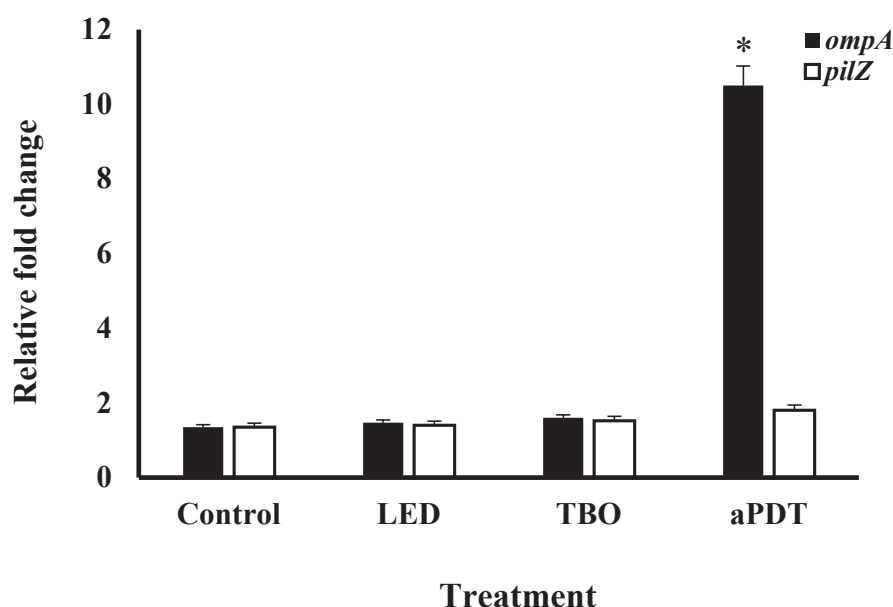


Figure 1: Effect of aPDT, TBO concentration and LED irradiation time on the virulence genes expression by colistin-resistant *A. baumannii* strain. Values are mean $\pm$ standard error of the mean of cDNA concentrations for each gene transcript from three replicate experiments. *, $P < 0.05$; significantly different from the control (strains grown without treatment).

ly, the compatibility of some clinical isolates of *A. baumannii* to all antibacterial drugs has led to appearance of polymyxin and tigecycline resisted PDR strains²⁰⁾. Many physicians are concerned about incurability of different bacterial infections²¹⁾. This concern in turn leads to greater efforts to find alternative methods to treat bacterial infections. In the recent decades, a lot of international research efforts are going to discover new antimicrobial agent. Nowadays, it is emphasized on elimination of possible MDR and how to take precautions against the establishment of MDR. New non-antibiotic approaches should be promoted in prevention and protection against infectious diseases and necessarily it should be considered as a significant priority in its research methods. In order to using aPDT techniques alone or in combination with antibiotics may be the basis for a new approach to reduce the rate of treatment failure in antibiotic treatment. Although a variety of studies^{22, 23)} shows inhibitory effect of aPDT on bacterial growth, little relevance is available about the effectiveness of these techniques on residual bacteria after low-dose radiation exposure in molecular and cellular levels.

Transcriptomic studies may lead to a better understanding of the molecular changes on the bacteria after aPDT. These tests lead to more efficient use of new techniques in medicine for treating antibiotic resistant infections.

Different light sources including coherent (lasers) and incoherent light sources have been used for irradiation in aPDT. The non-coherent light sources described in clinical studies on aPDT include the LED lamps, halogen lamps, and, more recently, intense pulsed light. Clinical application of LED light source seems to be simpler and cheaper, and field of illumination is larger. The spectra of the LED emission are relatively narrow and can correspond well with photosensitizers maximum absorption that results in time of irradiation can be shortened. In contrast to other incoherent light sources, LED is free of wavelengths that are not needed to achieve therapeutic properties. The other key fact of using LED sources is that they produce less heat than high-pressure lamps, are relatively inexpensive and highly durable in the long time²⁴⁾.

Gram-negative bacteria contain outer membrane vesicles [Omv] facilitating transfer of virulence factors into the host. OmpA is an outer membrane protein which is released by Omv. OmpA coding gene is one of the effective genes in formation of *A. baumannii* biofilm causing bacteria bind to the surface of epithelial cells⁶⁾.

This research shows that in comparison with group C, *ompA* gene expression has increased as in the first few min after radiation; it was reached to highest level. According to the fact that invasion to the constituents of cellular coverage is known as an effect of the aPDT method^{25, 26)} and considering our prior study²⁷⁾ about *lpsB* gene expression (It plays a role in lipopolysaccharide biosynthe-

sis)^{7, 28, 29)} which showed a significant increase after gene expression; it is consumed that free radicals produced by aPDT cause severe damage on *A. baumannii* outer membrane hence to avoid cellular lysis bacteria try to maintain the integrity of their outer membrane. Thus in order to repair the damages on outer membrane and maintenance of outer membrane integrity bacteria increases expression level of *ompA* gene.

Also, according to Dosselli. *et al.*³⁰⁾ research on *Staphylococcus aureus* showed that some proteins are fragmented following aPDT and many other after combination with oxygen conforms aggregates that will probably lead to the loss of cell function. Thus, in this study OmpA protein is probably damaged by oxygen radicals so in order to survive and organisms can repair damaged areas in outer membrane.

OmpA protein has a role in transition of antibiotics along the outer membrane and any changes in protein and decreased expression of *ompA* gene result in antibiotic (resistance to beta lactams) resistance³¹⁾. Therefore, according to damaged OmpA caused by aPDT, using combined therapy to remove infections caused by these bacteria would probably fail because damaged OmpA protein disturbs antibiotic entrance to *A. baumannii*. However according to Boluki *et al.* study³²⁾, it is concluded that OmpA damage wouldn't have effect on penetration of antibiotic agent to *A. baumannii* due to increased sensitization of bacteria to antibiotic which shows increased penetrance of antibiotic to bacteria.

OmpA is a virulence factor preventing *A. baumannii* death caused by immune system activation and complement attack⁶⁾. According to Kim *et al.* study³³⁾, OmpA inhibits function of the phagocyte cells of the host; therefore, it can be concluded that for the treatment of infections caused by aPDT immune system of body can possibly act more efficient because OmpA is damaged by aPDT.

While, many studies have associated the production of reactive oxygen species (ROS) with aPDT, the association between the significant rise of local ROS level and the exposure to LED light source and photosensitizers has not been assessed. In fact, ROS are formed as a toxic byproduct of the aPDT and have important roles in significant damage to microbial cell structures and as cell signaling. ROS are also implicated in positive effects on induction of SOS response³⁴⁾. The aPDT treatment expression of *ompA* was 8.7 folds higher than basal (**Fig. 1**); while, LED light source and photosensitizers treatment revealed no significant change in the expression of *ompA* expression. This suggests that stronger expression of *ompA* in aPDT treatment may be associated with ROS production. *ompA*, is an essential gene for the biosynthesis and integrity of outer membrane proteins. Increased transcription levels of *ompA* in the *A. baumannii* strains are consistent with the role of outer membrane in pro-

testing *A. baumannii* from adverse condition³⁵⁾. It is supposed that the critical role of *ompA* in bacterial outer membrane integrity maintenance, highlighted above, was corrupted due to interaction with ROSs produced followed by PDT treatment. Therefore, the SOS response in bacteria will lead to the expression of *ompA* to prevent the loss of outer membrane integrity and death under these circumstances.

Also, according to studies conducted^{6, 36)} indicated that OmpA protein plays a significant role in attachment of the bacteria to *Candida albicans* filament and human epithelial cells. It is known that in burn wound *A. baumannii* adheres to *C. albicans* filaments by the OmpA protein leading to development of robust biofilms on burn wound. Likely, OmpA damage under effect of aPDT leads to decreased adherence of bacteria to eukaryotic cells which prevents localization of bacteria at the site of infection. Consequently, infection will be treated more effectively.

Although *A. baumannii* does not increase motility rate in definite, it is able to spread rapidly over surfaces so that this motility is facilitated by type IV pili. PilZ has a main role in controlling type IV pili and producing biofilm^{7, 8)}. It was identified that expression of *pilZ* gene hasn't changed significantly following aPDT. According to our previous²⁷⁾ and current studies it has shown that the

most of gene expression is assigned to proteins such as LpsB and OmpA involving in maintaining outer membrane stability so it has been concluded that bacteria survives by using the most energy for repair of outer membrane so expression of *pilZ* gene is not required while exposing to oxygen free radicals.

Since a variety of biomolecules (lipids, amino acids, nucleotides, proteins) and cellular structures react with oxygen radicals^{37, 38)}, resistance to aPDT technique seems unlikely. Regarding limited access public to aPDT, and also requires an expert to perform aPDT, this treatment is limited to centers with specialized care. The most commonly used locally is the other limitation of this method and its use is difficult in systemic infections; that, of course, is not problematic about burn infections. Finally, when this technique is prescribed as a treatment, factors such as cost, and ease of management should be considered.

Conclusions

In summary, the main findings of this study revealed that aPDT as a supplement, likely decreases the failure rate in antibiotic therapy and can be effective in difficult situation such as antibiotic resistance.

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